Diurnal variation in the fraction of 3-hydroxy-3-methylglutaryl-coenzyme A reductase in the active form in the mammary gland of the lactating rat

Robin A. W. SMITH,* Bruce MIDDLETON* and David W. WEST†
*Department of Biochemistry, Queen's Medical Centre, Clifton Boulevard, Nottingham NG7 2UH, and
†Hannah Research Institute, Ayr, Scotland, KA6 5HL, U.K.

'Expressed' and 'total' activities of 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase) were measured in freeze-clamped samples of mammary glands from lactating rats at intervals throughout the 24 h light/dark cycle. 'Expressed' activities were measured in microsomal fractions isolated and assayed in the presence of 100 mM-KF. 'Total' activities were determined in microsomal preparations from the same homogenates but washed free of KF and incubated with exogenously added sheep liver phosphoprotein phosphatase before assay. Both 'expressed' and 'total' activities of HMG-CoA reductase underwent a diurnal cycle, which had a major peak 6 h into the light phase and a nadir 15 h later, i.e. 9 h into the dark period. Both activities showed a secondary peak of activity (around 68% of the maximum activity) at the time of changeover from dark to light, with a trough in the value of the 'expressed' activity that was close to the nadir value. 'Expressed' activity was lower than 'total' at all time points, indicating the presence of enzyme molecules inactivated by covalent phosphorylation. Nevertheless the 'expressed'/'total' activity ratio was comparatively constant and varied only between 43% and 75%. Immunotitration of enzyme activity, with antiserum raised in sheep against purified rat liver HMG-CoA reductase, confirmed the presence of both active and inactive forms of the enzyme and indicated that at the peak and nadir the variation in 'expressed' HMG-CoA reductase activity resulted from changes in the total number of enzyme molecules rather than from covalent modification. The sample obtained after 3 h of the light phase exhibited an anomalously low 'total' HMG-CoA reductase activity, which could be increased when Cl^- replaced F^- in the homogenization medium. The result suggests that at that time the activity of the enzyme could be regulated by mechanisms other than covalent phosphorylation or degradation.

INTRODUCTION

Extensive evidence in the literature supports the conclusion that the microsomal enzyme 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase [mevalonate:$NADP^+$ oxidoreductase (CoA-acylating), EC 1.1.1.34] catalyses the rate-determining step in cholesterogenesis in various mammalian tissues (Siperstein, 1970; Shapiro & Rodwell, 1971; Dietschy & Brown, 1974; Rodwell et al., 1976; Brown et al., 1979).

Hepatic reductase is known to exhibit a diurnal variation in 'total' activity throughout a 24 h light/dark cycle, in which its activity reaches a peak around the mid-point of the dark phase and attains a nadir at approximately the mid-point of the light phase (Hamprecht et al., 1969; Edwards et al., 1972; Shefer et al., 1972; Rodwell et al., 1976; Gibbons et al., 1983; Easom & Zammit, 1984). There is a parallel rhythm in the overall synthesis of cholesterol from acetate over the 24 h cycle in the liver which appears to arise from the rhythm in reductase activity (Back et al., 1969; Dugan et al., 1972). A correlation also exists between the rate of cholesterol biosynthesis and reductase activity for various other physiological states, such as starvation, cholesterol feeding and cholestyramine feeding (Kirsten & Watson, 1974; Dietschy & Brown, 1974).

Investigations on hepatic reductase have shown that two types of mechanism are operative in regulating its enzymic activity: firstly, long-term regulation, which may arise from a change in the quantity of enzyme protein by alteration in the rate of synthesis and/or degradation of the enzyme (Higgins & Rudney, 1973; Edwards & Gould, 1974; Beg & Brewer, 1981); secondly, acute regulation of its activity is thought to occur either by changes in membrane fluidity (Mitropoulous & Venkatesan, 1977) or by covalent modification involving reversible phosphorylation of the enzyme (Beg et al., 1973; Nordstrom et al., 1977; Beg et al., 1978; Gibson & Ingebritsen, 1978). Thus the enzyme exists in an active and an inactive form, the former being converted into the latter by a phosphorylation step catalysed by reductase kinase in the presence of MgATP. Experiments in vivo and in vitro subsequently confirmed that inactivation of reductase was due to phosphorylation of the enzyme itself (Beg et al., 1978; Keith et al., 1979; Beg et al., 1980; Gil et al., 1980, 1981) and that conversion of the inactive into the active form could be achieved by phosphoprotein phosphatase in the absence of either Mg^{2+} or ATP (Ingebritsen et al., 1981).

Immunotitration studies using specific HMG-CoA reductase antisera have been used to assess whether the normal diurnal rhythm in the activity of hepatic HMG-CoA reductase is a result of changes in the catalytic activity of pre-existing enzyme and/or of

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Abbreviation used: HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A.

changes in the quantity (concentration) of the enzyme. Conflicting results have been reported. Some investigators concluded that the changes in specific activity throughout the diurnal cycle were solely due to an alteration in the number of enzyme molecules, whereas others indicated that changes in enzyme quantity were accompanied by alterations in the catalytic efficiency of pre-existing enzyme (Hardgrave et al., 1979; Edwards et al., 1980; Kleinsek et al., 1980).

Little research has been carried out on HMG-CoA reductase in the mammary gland. A high activity of this enzyme has been observed in cultured explants of the rabbit mammary gland, with greater activity being present in the lactating gland than in pregnancy (Middleton et al., 1981). Evidence has also been presented that a diurnal variation in reductase activity exists in the lactating mammary gland of the rat (Gibbons et al., 1983). This cyclical variation bears a reciprocal relationship to that of the liver enzyme, since its nadir is at mid-dark.

The purpose of this present investigation was to confirm that this diurnal variation in reductase activity occurred in the lactating mammary gland, and to determine whether this rhythm was a consequence of changes in either the phosphorylation state of the enzyme, or alterations in the quantity of enzyme, or both.

MATERIALS AND METHODS

Treatment of animals

Pregnant rats of the Wistar strain (250–400 g), all of the same age, were housed in a light-controlled room (lights on 08:00–20:00 h) and maintained on Labsure diet and water ad libitum. At parturition the litter size was restricted to eight pups, and the mothers were then killed 10 days post partum at 11 different time points over the 24 h period (i.e. at 08:00, 10:00, 11:00, 14:00, 16:00, 17:00, 20:00, 21:00. 23:00, 02:00, 05:00 h). They were handled for several days before being killed to avoid stress on the day of death. For one group of animals the dam's food intake and pup weight gain were measured at 3 h intervals over 24 h.

Materials

DL-3-Hydroxy-3-methyl[3-^{14}C]glutaric acid and D-[2-^{3}H]mevalonolactone were obtained from Amersham International (Amersham, Bucks, U.K.).

Unlabelled HMG-CoA, mevalonolactone, NADP$^+$ (monosodium salt), glucose 6-phosphate (monosodium salt), glucose-6-phosphate dehydrogenase (type VII, from baker's yeast), pentobarbital, leupeptin and phenylmethanesulphonyl fluoride were obtained from Sigma (London) Chemical Co. (Poole, Dorset, U.K.).

For t.l.c., aluminium sheets (20 cm × 20 cm) pre-coated with silica gel 60 (layer thickness 0.2 mm) were purchased from BDH (Poole, Dorset, U.K.). 3-Hydroxy-3-methyl[3-^{14}C]glutaryl-CoA synthesized by the method of Williamson & Rodwell (1981) had a specific radioactivity of 26.5 d.p.m./pmol and was stored in small batches at −80 °C. For purposes of the reductase assay, the product was diluted with carrier substrate to give a final specific radioactivity of 5.3 d.p.m./pmol.

Preparation of phosphoprotein phosphatase

Protein phosphatase was prepared from sheep liver essentially by the method of Brandt et al. (1975) up to

and including the first DEAE-cellulose step. The product, which had a specific activity of 112.7 units/mg of protein, was stored in 60% (v/v) glycerol at −20 °C. A unit corresponds to the release of 1 μmol of P$_i$/min at 30° C.

Purification of rat liver HMG-CoA reductase and generation of antiserum

HMG-CoA reductase was purified to near homogeneity from rat liver by the procedure of Ness et al. (1979), by using the solubilization procedure suggested by Edwards et al. (1979) and a previously reported heat-treatment step (Beg et al., 1980). Male Wistar rats were treated with mevinolin (30 mg/kg body wt.) 17 h before death to increase the yield of enzyme (Endo, 1981). The final product was purified 8176-fold and had a specific activity of 11528 nmol/min per mg of protein, comparable with the value obtained by Edwards et al. (1980). SDS/polyacrylamide-gel electrophoresis revealed the presence of four protein bands, the major band having a molecular mass corresponding to 53000 Da, in agreement with previously reported values (Kleinsek et al., 1977; Srikantaiah et al., 1977; Ness et al., 1979; Edwards et al., 1980). On non-denaturing electrophoretic gels the enzyme activity co-migrated with the major protein band. The purified enzyme was stored at −20 °C in 50% (v/v) glycerol.

Antibodies to this product were raised in sheep after intramuscular and subcutaneous injection. Antiserum was prepared from blood drawn 9 weeks after the initial injection.

The crude antiserum was stored in small batches at −80 °C. Immunoblotting showed that it was the 53000 Da fragment of the purified enzyme that reacted with the antiserum.

Preparation of mammary-gland microsomes (microsomal fractions)

The abdominal mammary glands of anaesthetized (pentobarbital; 60 mg/kg body wt.) lactating rats were exposed, and both were rapidly freeze-clamped, by using metal tongs which had been pre-cooled in liquid N$_2$. The frozen glands were then stored in liquid N$_2$ before preparation of the microsomal fraction.

Frozen mammary tissue (2 g) was quickly broken into small pieces on a metal surface cooled to liquid-N$_2$ temperature and homogenized (Polytron; 10400 rev./min, 30 s) in 5 vol. of ice-cold medium containing 100 mM-KF, 50 mM-KH$_2$PO$_4$, 0.3 M-sucrose, 10 mM-EDTA, 1 mM-EGTA, 25 mM-2-mercaptoethanol, 1 mM-phenylmethanesulphonyl fluoride and 50 μM-leupeptin at pH 7.0. The resulting homogenate was centrifuged (10000 $g_{av.}$, 15 min, 4 °C) to obtain a post-mitochondrial supernatant, which was divided into two portions before both were centrifuged at high speed (100000 $g_{av.}$, 60 min). One of the resultant pellets was resuspended in the homogenization medium, and the second was resuspended in a similar medium in which the fluoride had been replaced by KCl. Both were again centrifuged (100000 $g_{av.}$, 45 min) to obtain microsomal pellets. The fluoride-treated pellet was resuspended in a medium of the following composition (5 mg of microsomal protein/ml): 0.1 M-sucrose, 100 mM-KF, 10 mM-EDTA, 40 mM-KH$_2$PO$_4$, 50 mM-KCl, 1 mM-EGTA, 5 mM-dithiothreitol, 1 mM-phenylmethanesulphonyl fluoride, 50 μM-leupeptin, at pH 7.2, and

the second, fluoride-free, pellet was resuspended in a similar medium in which KCl replaced the KF.

Assay of HMG-CoA reductase activity

'Expressed' activity. HMG-CoA reductase in the microsomes was routinely assayed, at two different protein concentrations, essentially by the method of Balasubramaniam et al. (1976). The assay system contained 100 μg or 200 μg of fluoride-treated microsomal protein, [3-^{14}C]HMG-CoA (20 nmol; 5.3 d.p.m./pmol), 20 mM-glucose 6-phosphate, glucose-6-phosphate dehydrogenase (3 units/ml), 2.5 mM-NADP$^+$, 5 mM-dithiothreitol, 0.1 M-sucrose, 100 mM-KF, 30 mM-EDTA, 40 mM-KH$_2$PO$_4$, 50 mM-KCl, 1 mM-EGTA, 1 mM-phenylmethanesulphonyl fluoride, 50 μM-leupeptin, pH 7.2, in a total volume of 150 μl. After 10 min preincubation in the absence of, and then a further 5 min in the presence of, the NADP-generating system, the reaction was initiated by the addition of [3-^{14}C]HMG-CoA and terminated after 15 min at 37 °C by the addition of 10 M-HCl (10 μl). [^{3}H]Mevalonolactone (44400 d.p.m.) was then added as an internal standard, and the tubes were further incubated at 37 °C for at least 30 min to ensure complete lactonization. The denatured protein was removed by centrifugation at 2000 g for 2 min and the mevalonolactone formed in the supernatant isolated by t.l.c. [silica gel, developed with toluene/acetone (1:1, v/v)]. The ^{14}C incorporated into mevalonolactone was measured by using the recovery of the added [2-^{3}H]mevalonolactone to correct for losses. The product was located by observation, under u.v. light, of a concentrated standard of mevalonolactone run in parallel on the same plate (R_F 0.35–0.65). The region corresponding to mevalonolactone was cut from the plates, added to 10 ml of scintillant, and the radioactivity was determined in a Packard Tri-Carb scintillation counter. Recoveries of 70–90% were routinely observed. The formation of mevalonolactone under the conditions used was linear for up to 20 min and up to 400 μg of microsomal protein. Controls with the complete system but without microsomes, and with the complete system but without the NADPH-generating system, were assayed to correct for any HMG-CoA lyase and cleavage-enzyme activity in the preparation. The K_m for the mammary-gland HMG-CoA reductase was determined to be 5.2 μM-HMG-CoA and the $V_{max.}$ to be 2.38 nmol/min per mg of microsomal protein.

'Total' activity. Dephosphorylation and concomitant activation of the enzyme was achieved by incubating fluoride-free microsomes (100 μg) with 3.3 units of sheep liver phosphoprotein phosphatase at 37 °C, initially for 10 min and then for a further 5 min after the addition of the NADPH-generating cocktail, before initiation of the assay by [3-^{14}C]HMG-CoA. The specific radioactivity was then determined under the same conditions as were used for the fluoride-treated microsomes, except that KCl was replaced by KF in the assay mixture.

Units. One unit of enzyme activity was defined as 1 nmol of mevalonate formed/min.

Immunochemical studies

Immunotitration of the mammary-gland HMG-CoA reductase was carried out essentially by the method
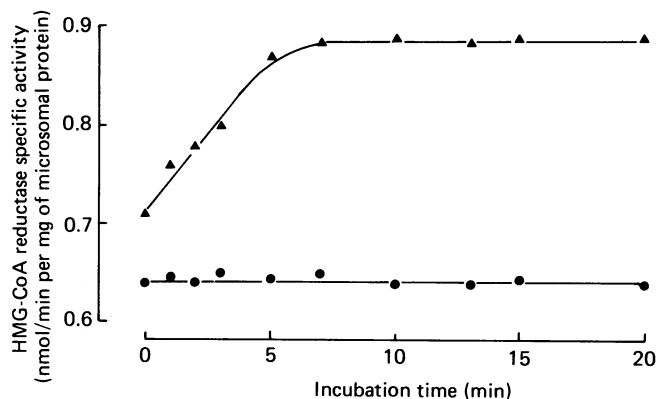
Fig. 1. Time course of activation of HMG-CoA reductase in rat mammary-gland microsomes by exogenous partly purified sheep liver protein phosphatase

Microsomes prepared from the mammary gland of a rat killed 6 h into the light phase were incubated with protein phosphatase in the presence of 100 mM-KCl (▲) or 100 mM-KF (●). At the intervals shown, samples of the incubation mixtures were assayed for HMG-CoA reductase activity.

described by Hardgrave et al. (1979), with the crude sheep antiserum raised against rat liver HMG-CoA reductase. Immunotitrations, using constant amounts of enzyme activity and increasing amounts of antibody, were carried out on microsomes which had been frozen and thawed only once. Fluoride- and phosphatase-treated microsomes (225 μg, 45 μl) were preincubated with an equal volume of antiserum of various dilutions (1:2 to 1:256) for 30 min at 37 °C. HMG-CoA reductase assays were then carried out for 15 min to measure the residual enzyme activity. The specificity of the antiserum was tested in three ways.

(i) Presorbed reductase antiserum was prepared by incubation of reductase antiserum with purified rat liver HMG-CoA reductase for 60 min at 37 °C, followed by a 48 h period at 4 °C. The immunoprecipitate was removed by centrifugation (5 min at 2000 $g_{av.}$) and the supernatant used for immunotitration. Such presorbed antisera did not cause a diminution in enzyme activity.

(ii) No neutralization of enzyme activity was observed when microsomes were titrated with control serum obtained from sheep before injection with HMG-CoA reductase antigen.

(iii) Ouchterlony double-diffusion analysis demonstrated the presence of only a single immunoprecipitation line.

Thus the loss of enzyme activity was a result of direct reaction of the antibody on the active site of the HMG-CoA reductase molecule.

Assay of arylesterase

The activity of arylesterase in both the homogenate and the microsomal fraction was determined by the method of Shephard & Hubscher (1969).

Protein determination

Protein concentration was determined by the dye-binding method of Bradford (1976), with bovine serum albumin as a standard.
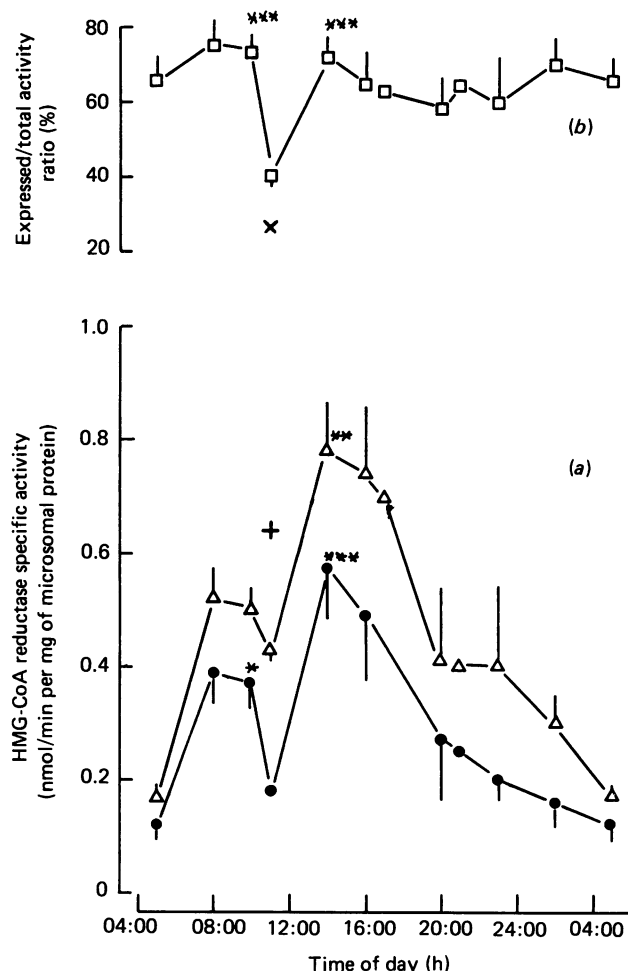
Fig. 2. Diurnal variation (a) in total (△) and expressed (●) activities of HMG-CoA reductase and (b) in the fraction of the enzyme in the active form (□) in the lactating rat mammary gland

Values are means ± s.e.m. for three to eleven determinations performed on microsomal preparations of lactating mammary glands obtained from separate rats. Where no error bars are indicated, the errors were too small to be shown. The fraction of HMG-CoA reductase in the active form is given as the expressed/total activity (percentage). The calculated values for the total activity and the expressed/total activity ratio at 11:00 h adjusted for the low value of the Equivalence Point (see the text) are marked (+ and × respectively). Statistical significance of differences between time points indicated and the 11:00 h time point: ***$P < 0.001$, **$P < 0.01$, *$P < 0.02$ (determined by Student's t test).

RESULTS AND DISCUSSION

The time course of the phosphatase-induced activation of HMG-CoA reductase in mammary-gland microsomes in the absence of fluoride is shown in Fig. 1. Activation was complete within 10 min at 37 °C. In the presence of KF no activation of enzyme activity was observed by this phosphatase treatment. Preincubation of microsomes with the sheep liver phosphoprotein phosphatase for this time period, before addition of the NADPH-regenerating system, was routinely used throughout this study to obtain 'total' enzyme activity, defined as the activity of HMG-CoA reductase after full activation (dephosphoryl-

ation) in the absence of fluoride but in the presence of EDTA. In contrast, the 'expressed' enzyme activity refers to the activity of HMG-CoA reductase when isolated in a medium containing fluoride (which inhibits endogenous phosphoprotein phosphatase activity) and EDTA (to block cellular protein kinases). This gives the activity of the enzyme actually expressed *in vivo* at the time that the tissue was isolated. By using the freeze-clamping technique, both 'expressed' and 'total' HMG-CoA reductase activity of the lactating mammary gland could be determined. The results of such measurements carried out at 11 time points throughout the light/dark period are shown in Fig. 2(a). The 'total' activity of the mammary gland HMG-CoA reductase exhibited a diurnal variation, with a pronounced peak of activity, 0.78 ± 0.09 unit/mg of protein ($n = 11$) at 14:00 h, 6 h into the light phase, and a minimum of 0.17 ± 0.02 unit/mg of protein ($n = 5$) occurring at 05:00 h, 9 h into the dark period, a 4.6-fold difference. The 'expressed' enzyme activity showed a similar diurnal rhythm, with a peak value of 0.57 ± 0.09 unit/mg of protein ($n = 11$) at 14:00 h and a minimum of 0.12 ± 0.03 unit/mg of protein ($n = 5$) at 05:00 h, a 4.8-fold change. 'Expressed' activities were always lower than 'total' activities throughout the 24 h, indicating that a proportion of the enzyme molecules present in all the extracts were inactive and could be re-activated by phosphatase treatment. 'Total' enzyme activities determined for non-frozen mammary tissue at 12:00 h and at 24:00 h agreed with the results obtained from freeze-clamped samples (results not shown), confirming that freeze-clamping and subsequent storage of samples did not cause loss of enzyme activity. The specific activity of arylesterase, a microsomal marker enzyme, was also determined in both the homogenate and the microsomal fraction. The activity of this enzyme in the microsomal fraction could then be expressed as a fraction of that present in the original homogenate. This relationship could be applied to the HMG-CoA reductase activity in the microsomes giving an estimate of the enzyme activity present in the homogenate. Calculated in this way, the total HMG-CoA reductase activity in the homogenate at 11:00 h was 0.31 unit/mg of homogenate protein, which is more than twice the value reported by Gibbons *et al.* (1983) for this time point.

As well as the peak of activity at 14:00 h, both diurnal rhythms indicated that a complicated pattern existed across the first few hours of the light period, such that the rise from nadir to peak occurred in two stages. 'Total' activity at 08:00 h was determined to be 0.52 ± 0.05 unit/mg of protein ($n = 4$), but this had declined to 0.43 ± 0.02 unit/mg of protein ($n = 9$) by 11:00 h before rising rapidly to the peak at 14:00 h. This pattern was even more pronounced with the 'expressed' activity, which decreased from 0.39 ± 0.06 unit/mg of protein ($n = 4$) at 08:00 h to 0.18 ± 0.01 unit/mg of protein ($n = 9$) at 11:00 h.

From these results the ratio of expressed total activity at specific intervals over the 24 h cycle could be calculated (Fig. 2b), giving the fraction of the enzyme population in the active form. The results indicated that in general the ratio remained fairly constant, varying only between 59% and 70% over most of the 24 h, with extreme values of $40.6 \pm 2.8\%$ ($n = 9$) and $75.0 \pm 7.1\%$ ($n = 4$) being recorded. This contrasted with the liver enzyme, which showed a much greater variation in this
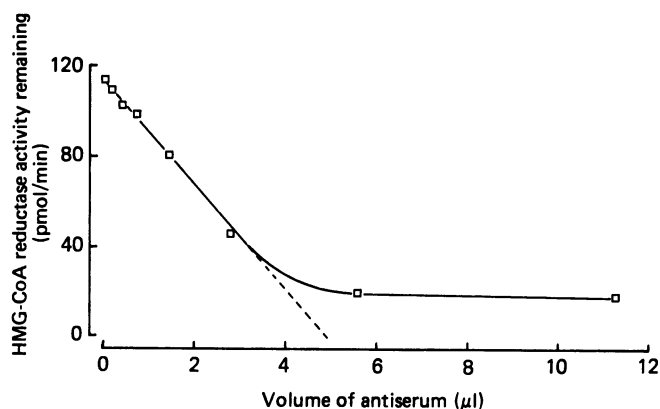
Fig. 3. Immunotitration of HMG-CoA reductase activity

The Figure shows the typical result obtained when the HMG-CoA reductase activity present in microsomes isolated from the lactating mammary gland of a rat, killed at 14:00 h, was titrated with antiserum. The Equivalence Point (E.P.) is given by the slope of the linear portion of the graph. For further details see the Materials and methods section.

ratio, of 28–80% (Easom & Zammit, 1984). Moreover, the extreme values noted for the mammary-gland reductase did not coincide with the nadir and peak of the diurnal cycle, as was the situation with the liver. Instead, the largest variation occurred across the first few hours of the light period, when the ratio fell sharply, from $73.6 \pm 4.5\%$ ($n = 3$) to $40.6 \pm 2.8\%$ ($n = 11$) between 10:00 and 11:00 h, and increased steeply again to $71.9 \pm 5.1\%$ ($n = 11$) at 14:00 h.

Immunotitration

The crude antiserum raised in sheep against the purified rat liver HMG-CoA reductase neutralized the HMG-CoA reductase activity present in the microsomes obtained from the lactating mammary glands. A typical immunotitration plot of enzyme activity versus quantity of antiserum is presented in Fig. 3. The linear diminution to 50% of HMG-CoA reductase activity was followed by a more gradual loss of activity. This type of inactivation has been reported for this enzyme in other tissues (Kleinsek et al., 1980; Edwards et al., 1980; Lehoux et al., 1985).

Equivalence points

The equivalence point (E.P.) reflects the ratio of active to inactive molecules present in the sample and was defined as the enzyme activity (in pmol of mevalonate produced/min) neutralized by 1 μl of antiserum. This was calculated by linear regression analysis of the critical portion of the curve obtained when enzyme activity was plotted against quantity of antiserum (Fig. 3). Differences in E.P. between two samples represents a change in the number of active molecules, a smaller E.P. indicating a decrease in the number of active molecules present. This inactivation may arise from a number of enzyme modifications, including, in the context of this report, covalent modification by phosphorylation. Although the E.P. is not directly related to the number of enzyme molecules present, it can be linked to that parameter by using the specific enzyme activity (S.A.) of the sample. It is thus possible to determine whether the change in enzyme activity between two samples is due to an alteration in the number of enzyme molecules, to a variation in the activity for other reasons, or both. If the E.P. does not differ between two samples, then any alteration in activity is solely due to a change in enzyme quantity. If both the E.P. and the S.A. values of the two samples are altered, then two possibilities exist. (a) If the ratio of the two E.P. values is identical with the ratio of the two S.A. values, then the change in activity results for reasons other than a change in enzyme quantity, e.g. a variation in the phosphorylation status. (b) If the ratio of the two E.P. values differs from that of the two S.A. values, then the change is due to both parameters, i.e. phosphorylation status and enzyme quantity. Dividing the ratio of S.A. values by the ratio of E.P. values gives an indication of the proportionate change in the number

Table 1. Specific activities and equivalence points for HMG-CoA reductase activities for rat mammary-gland microsomes

Microsomes were obtained from the mammary glands of rats killed at 11:00, 14:00 and 02:00 h time points in the light/dark cycle. HMG-CoA reductase activities and immunotitration data were determined on fluoride-free (total activity) and fluoride-treated ('expressed' activity) microsomes (see the Materials and methods section for full details). The results are mean values $\pm$ s.e.m. for the numbers of determinations shown in parentheses. Statistical significances of differences in specific activities and equivalence points between 11:00 h and the times indicated are ***$P < 0.001$, **$P < 0.01$. Statistical significances of differences between the ratio of specific activities and the ratio of equivalence points are also shown: ††$P < 0.01$, N.S., not significant.

|	Fluoride-free microsomes		Fluoride-treated microsomes			
Time of death (h)	'Total' specific activity (nmol/min per mg of protein) (SA$_t$)	Equivalence point (pmol/min per μl of antiserum) (EP$_t$)	'Expressed' specific activity (nmol/min per mg of protein) (SA$_e$)	Equivalence point (pmol/min per μl of antiserum) (EP$_e$)	Ratio of specific activities (SA$_e$/SA$_t$)	Ratio of equivalence points (EP$_e$/EP$_t$)
11:00	0.43 ± 0.02 (9)	20.0 ± 2.0 (3)	0.18 ± 0.01 (9)	16.0 ± 1.0 (3)	0.43 ± 0.01 (3)	0.81 ± 0.05 (3)††
14:00	0.78 ± 0.09 (11)***	29.0 ± 1.0 (5)***	0.57 ± 0.09 (11)**	23.0 ± 3.0 (5)	0.69 ± 0.09 (5)	0.79 ± 0.07 (5) N.S.
05:00	0.17 ± 0.02 (5)**	29.0 ± 1.0 (5)***	0.12 ± 0.03 (5)	23.0 ± 4.0 (5)	0.65 ± 0.07 (5)	0.77 ± 0.11 (5) N.S.
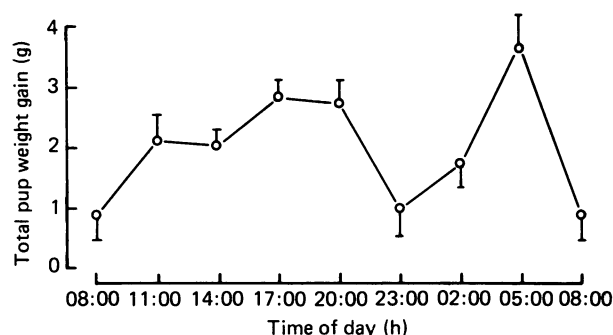
Fig. 4. Cyclical variation in total pup weight gain

The total weight of all eight pups of each litter was determined at the time points shown. The results are the means±s.e.m. for 19 litters and indicate the weight gain during the previous 3 h.

Fig. 5. Cyclical variation in food intake of lactating rats

The food intake was measured at the points shown and represents the food consumed during the previous 3 h. The results are the mean values±s.e.m. for 19 lactating rats.

of enzyme molecules, e.g. with S.A. ratio of 3.0 and an E.P. ratio of 2.0, 3.0/2.0 = 1.5, indicating a 50% increase in enzyme protein.

The results obtained on immunotitration of the HMG-CoA reductase activity present in the mammary microsomes from rats at the peak (14:00 h) and the nadir (05:00 h) of the diurnal cycle together with those obtained from rats at 11:00 h are shown in Table 1. The fluoride-treated and fluoride-free microsomes, obtained at the same time point, gave different equivalence points, indicating that a proportion of the fluoride-treated enzyme molecules was inactive, thus providing confirmation of the existence of both active and inactive forms of the enzyme. When different time points were considered, i.e. 14:00 and 05:00 h, the equivalence points for HMG-CoA reductase in the fluoride-treated microsomes were identical (Table 1). This indicates that at these two points the same ratio of active to inactive enzyme molecules existed and the difference in expressed activity was solely due to alteration in the total number of enzyme molecules and no change in phosphorylation status was involved. The equivalence point for the enzyme present in the fluoride-containing microsomes at 11:00 h was even lower than at 14:00 or 05:00 h. An increased proportion of inactive molecules in comparison with the peak and nadir microsomes was thus indicated. However, the fluoride-free microsomes obtained from rats at 11:00 h, when treated with phosphatase, gave an unexpectedly low equivalence point for the enzyme, 20.0 instead of the expected 29.0 (pmol/min per μl of antiserum). That would indicate that the microsomes contained inactive (or crippled) molecules that were not re-activated by the phosphatase treatment. Fluoride itself did not interfere with the immunotitration of enzyme activity, since the ratio of HMG-CoA reductase specific activities from microsomes prepared in the presence or absence (and subsequent phosphatase treatment) of 100 mM-fluoride at 14:00 and 05:00 h was similar to the corresponding ratio in equivalence points for the enzyme (Table 1).

Food intake and pup weight change

The function of the HMG-CoA reductase in the mammary gland is to provide substrate for the synthesis of cholesterol (and other isoprenoids) for secretion into milk and for replenishment of cellular membranes.
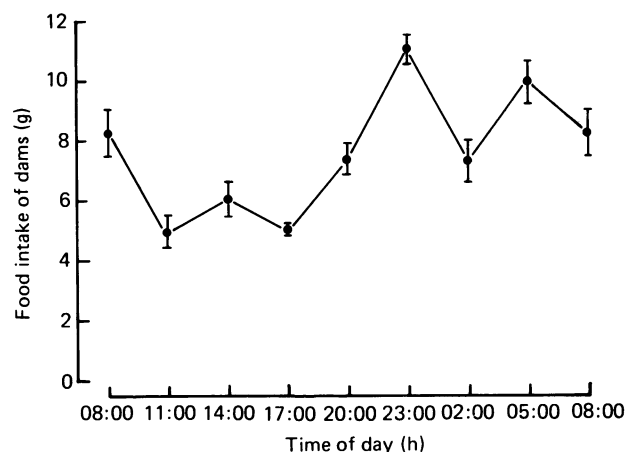
Excess cholesterol causes a feedback inhibition of HMG-CoA reductase (Brown & Goldstein, 1980; Ingebritsen & Gibson, 1980), so that the activity of the mammary enzyme should be very responsive to the removal of cholesterol by the suckling of the pups, which regulates the demand for milk and synthesis of milk components.

Both pup weight change and the mother's food intake were measured at 3 h intervals throughout the 24 h cycle, and the results obtained are presented in Figs. 4 and 5 respectively. The times indicated are those at which the pups and the food were weighed, and the weight change is the average gained by the pups, or food consumed by the mother, in the 3 h since the last observation. The pup-weight-gain data indicated a bimodal distribution during the 24 h, with a steady weight gain being exhibited during the light period which was rapidly curtailed during the first 3 h of darkness. The following 6 h showed a very rapid increase in weight, with 38% of the weight gain for the 24 h period being accumulated in this period. In spite of these variations, the weight gained by the litter was quite evenly spread over the 24 h, with 57% being gained in the light period and 45% gained in the dark period.

The lactating rat has a much increased dietary intake in relation to the virgin female (Fell et al., 1963; Cripps & Williams, 1975; Munday & Williamson, 1983) and the total daily intake for the rats in our study averaged 60.0 g, in fairly good agreement with the results of Munday & Williamson (1983). However, between 20.00 and 08.00 h the lactating rats in the present study continued to exhibit the bimodal feeding pattern associated with male rats (Siegel, 1961) and virgin females (Tachi et al., 1981) rather than the single peak of food intake reported by Munday & Williamson (1983). The result was that 39% of the food intake was consumed during the light phase, compared with the 35% eaten in the same period reported by Munday & Williamson (1983).

General discussion

The diurnal rhythm in total HMG-CoA reductase activity seen in the freeze-clamped samples of mammary

Table 2. Influence of fluoride on the 'total' HMG-CoA reductase activity of the lactating rat mammary gland

Mammary glands, freeze-clamped at the times shown, were homogenized, as detailed in the Materials and methods section, in media containing either 100 mM-KF or 100 mM-KCl. Microsomes were isolated from each homogenate and both were washed with KCl-containing medium to remove fluoride ions. The 'total' HMG-CoA reductase activity was determined after each microsomal pellet was incubated with phosphoprotein phosphatase. The results are means$\pm$S.E.M. for the numbers of determinations shown in parentheses. Statistical significances of differences in both specific activities and equivalence points when homogenized in KF and KCl are indicated; $**P < 0.001$, $*P < 0.02$ (determined by application of Student's t test); N.S., not significant.

Homogenization in ...	KF		KCl	
Time of death (h)	Specific activity (unit/mg of protein)	Equivalence point (pmol/min per μl of antiserum)	Specific activity (unit/mg of protein)	Equivalence point (pmol/min per μl of antiserum)
11:00	0.43 ± 0.02 (9)	20.0 ± 2.0 (3)	0.65 ± 0.03 (4)**	29.7 ± 0.7 (3)*
14:00	0.78 ± 0.09 (11)	29.0 ± 1.0 (5)	0.73 ± 0.04 (3) N.S.	30.0 ± 2.0 (3) N.S.

tissue was broadly in agreement with that reported by Gibbons et al. (1983) in that the peak of activity occurred during the daylight hours and the nadir during the dark period. The activity pattern of the mammary-gland enzyme was thus confirmed to be the inverse of the pattern seen in the liver. Since 60–70% of the cholesterol output into milk is supplied from exogenous sources (Clarenburg & Chaikoff, 1966; Gibbons et al., 1983), from either the liver or the intestine, it should be possible to correlate the variations in mammary-gland HMG-CoA reductase activity with the diurnal variation of the hepatic enzyme (Easom & Zammit, 1984), the food intake of the mothers and the cholesterol synthesized in the gland. In fact, consideration of the cholesterol synthesized in the gland, as determined by Gibbons et al. (1983), indicates that HMG-CoA reductase activity only correlates with synthesis of the sterol across the hours of darkness when both the enzyme activity and the cholesterol synthesized in the gland were declining. During this period, when the animals are normally most active, the dam's food intake was greatest and the activity of the hepatic HMG-CoA reductase was at its peak. The cholesterol requirements of the gland could be increasingly satisfied by cholesterol from the liver and from the intestine and the necessity for active HMG-CoA reductase in the mammary gland correspondingly decreased. At the same time lipogenesis in the mammary gland was increasing (Munday & Williamson, 1983), providing greater competition for substrates common to both pathways.

A correlation between cholesterol synthesis and HMG-CoA reductase activity in the gland does not, however, exist over the daylight hours. During the light period the food intake of the dams was decreased to about 50% of that eaten during the dark period, and hepatic HMG-CoA reductase fell to a minimum so that supply of exogenous cholesterol to the gland would have been decreased. Pup weight gain was, however, still substantial, indicating that removal of milk continued to place a demand on the gland for cholesterol. Nevertheless, in spite of maximal HMG-CoA reductase in the tissue, cholesterol synthesis by the gland was reported to be at a minimum. Lipogenesis also fell to its lowest value during this time, so increased competition from this pathway cannot explain the increased HMG-CoA

reductase activity. This paradoxical situation did not occur in isolated mammary acini, where, although lipogenesis and HMG-CoA reductase activity continued to exhibit the same reciprocal relationship as observed in vivo, cholesterol synthesis closely paralleled the changes in HMG-CoA reductase activity (Smith et al., 1986). It would appear that only in the situation in vivo does the relationship between HMG-CoA reductase activity and sterol synthesis break down. The high enzyme activity in the gland is puzzling, but decreased substrate supply to the mammary gland, requiring a high activity of HMG-CoA reductase to compete effectively with other pathways, may be an explanation (Gibbons et al., 1983).

The most important feature of the results presented here is the demonstration that the activity of the HMG-CoA reductase in the lactating rat mammary gland is, like the hepatic enzyme (Easom & Zammit, 1984), regulated both by enzyme turnover and by phosphorylation. However, unlike the hepatic enzyme, the degree of activation by the protein phosphatase treatment varied comparatively little with the time at which the microsomes were obtained from the rats. The greater proportion of the enzyme molecules present were in the active form throughout the 24 h and the activation achieved by phosphatase treatment was generally less than 2-fold. The result was that the 'expressed' activity increased only 4.7-fold from nadir to peak, compared with a 12-fold activation observed for the hepatic HMG-CoA reductase (Easom & Zammit, 1984). The exception to this generality occurred around 11:00 h, when the phosphorylation status of the enzyme showed marked changes over a short period. Even here the activation achieved was only 2.4-fold. At this time the enzyme activity expressed in the gland was decreased to almost the same value as was observed at the nadir. Interestingly, cholesterol synthesis was also stated to be at a minimum at this time (Gibbons et al., 1983). The values recorded for both total and expressed activity at 11:00 h were particularly low, and it is difficult to suggest a physiological explanation for such a sharp decrease in enzyme activity at this time. Immunotitration indicated that after activation by phosphatase the enzyme at 11:00 h had an equivalence point low in comparison with the value obtained at 14:00 and

05:00 h, i.e. 20.0 instead of 29.0 pmol/min per μl of antiserum. The possibility exists that inactivation of the enzyme by means other than by covalent phosphorylation had occurred at that time point. If the value of the total activity recorded at 11:00 h was adjusted by a factor of 29/20, a calculated value of 0.63 unit/mg of protein was obtained (Fig. 2), a value which makes the diurnal pattern correspond more closely with the results reported previously (Gibbons et al., 1983). Gibbons et al. (1983) homogenized their tissue in a chloride-containing medium and incubated the homogenate before assaying HMG-CoA reductase activity, whereas we have routinely used medium containing 100 mM-KF to inhibit phosphatases. Although fluoride is known to inhibit phosphatases, there remained the possibility that the presence of this ion had other effects. This was confirmed when mammary-gland samples freeze-clamped at 11:00 h were homogenized in a medium in which Cl$^-$ ions replaced the usual fluoride. Subsequent assay of the resultant HMG-CoA reductase activity gave a value of 0.65 unit/mg of protein and immunotitration indicated an E.P. of 30.0 pmol/min per μl of antiserum, results essentially identical with the calculated values (Table 2). The enzyme was phosphorylated to its greatest extent at this time point, and it could be argued that the re-activation by phosphatase treatment would take longer than normal, and that this might provide an explanation for the lower value of 'total' activity obtained. Investigation of the re-activation, with sheep liver phosphatase, of a sample of microsomes from an animal killed at 11:00 h indicated that the normal time course was operative. Furthermore, a sample of microsomes, obtained at 11:00 h but containing HMG-CoA reductase 98% phosphorylated, showed the same 'total' activity, after phosphatase treatment, as normal microsomes isolated at this time. Thus delayed re-activation was not a tenable explanation for this observation. Calculation of the 'expressed' activity at 11:00 h, as a percentage of this new value for 'total' HMG-CoA reductase activity gave a value of 27% compared with the 40.6% previously calculated, indicating an even greater change in enzyme activity at this point (Fig. 2b) and making the overall variation in 'expressed' activity much closer to that observed with the hepatic enzyme.

Glands clamped at 14:00 h did not show this phenomenon, since no significant change in the 'total' HMG-CoA reductase activity was observed when they were homogenized in Cl$^-$-containing medium (Table 2). It would appear that, for the mammary glands sampled at 11:00 h, the presence of F$^-$ in the medium had resulted in a lower 'total' enzyme activity, the reasons for which are not obvious. F$^-$ is known to have a slightly stronger 'salting-out' effect than Cl$^-$ (Hatefi & Hanstein, 1969), so the use of F$^-$ may have resulted in the release of factors from the microsomes which prevented full activation, although why this should only apparently occur at this particular time point is obscure. This possibility requires further investigation, as does the effect of fluoride at other time points.

The present studies nevertheless indicate that there are marked diurnal changes in the activity of HMG-CoA reductase in the lactating mammary gland of the rat and that the variation in expressed activity, although generally following closely that of total activity, does differ from it substantially at a number of time points.

They also suggest that in the early phase of the light period the activity of the enzyme is regulated by other mechanisms as well as by covalent phosphorylation and by degradation.

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